

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**Applicant:** Kwon, Byoung  
**Ser. No.:** 08/955,572  
**Filed:** October 22, 1997  
**Title:** Human H4-1BB Protein

**Examiner:** Robert S. Landsman  
**Group Art Unit:** 1647  
**Atty. Docket:** 29920-77259

---

**AFFIDAVIT FOR PRIORITY SHOWING UNDER 37 CFR 41.202(d)(1)**

Commissioner of Patents  
P.O. Box 1450  
Alexandria, Virginia 22313-1450

Sir:

I, Byoung Kwon declare and state as follows:

1. I am the named inventor of the subject matter claimed in the above-identified patent application, U.S. application Serial No. 08/955,572, filed on October 22, 1997. The pending claims relate to human 4-1BB proteins and fragments.
2. I acknowledge that under an agreement with the owner of the subject invention I will receive a percentage of any royalties generated from my invention.
3. I received a Certificate in 1968, a D.D.S. in 1972, and a M.S. in Microbiology in 1974, from Seoul National University, Seoul, Korea. In 1981, I received a Ph.D. in Microbiology from the Medical College of Georgia, Augusta, Georgia. From 1981-1984, I was a postdoctoral fellow in the Department of Human Genetics at Yale University School of Medicine, New Haven, Connecticut. I was the Head of Medical Genetics at the Guthrie Research Institute, Sayre, Pennsylvania, from 1984-1988. From 1988-1993, I was an Associate Professor in the Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana. From 1993-1999, I was a Professor in that same Department. I am currently a Professor of Ophthalmology at Louisiana State University and also a Director of The Immunomodulation Research Center and Department of Biological Sciences, University of Ulsan, Ulsan, Korea. I have authored or co-authored over 250 papers, primarily in the areas of the identification and characterization of molecules involved in lymphocyte activation and proliferation, and the molecular basis for pigmentation.

Byoung S. Kwon

08/948,764

4. I have reviewed the Goodwin *et al.* patent (U.S. Patent No. 7,211,259) (the "Goodwin patent") cited by the Examiner in the Office Action dated March 24, 2008 and provide this affidavit in support of priority showing for the patentability of pending claims 5, 6, 24, and 26-31 of the application referenced above.
5. I understand that the priority application resulting in the Goodwin patent was filed May 7, 1993 and therefore the effective date of the Goodwin patent is May 7, 1993.
6. Prior to the May 7, 1993 filing date of the patent application to the Goodwin *et al.* patent, I had isolated and purified at least a portion of a human 4-1BB (H4-1BB) gene and thereafter proceeded diligently to characterize the full length gene. Also, prior to May 7, 1993, I had prepared DNA constructs encoding human 4-1BB proteins that included the extracellular domain of H4-1BB.
7. Prior to May 7, 1993, I had conceived of isolating the human homolog of 4-1BB gene as evidenced by the grant proposals that were submitted based on my research with mouse 4-1BB gene and its gene product. Attached to this affidavit are EXHIBITS 1-3 (KWON000001-9) illustrating the development of mouse 4-1BB research that served as a basis for the conception of isolating human 4-1BB gene homolog. As stated in EXHIBIT 1, I conceived of experiments that involved expression of T-cell specific cDNA and raising antibodies against the expressed protein products of 4-1BB. (KWON000001). Prior to May 7, 1993, I analyzed several sequences of cDNA clones and compared the sequences to those available in the GenBank. (KWON000003-7). I sequenced 14 novel cDNAs from mouse, and one of which was later designated as 4-1BB. (KWON000007).
8. As stated in EXHIBIT 3, prior to May 7, 1993 I expressed as one of my long-term objectives the need to "identify the human homologue of each of the molecules and to seek clinical applications for human immunodeficiency and other diseases such as human malignancies and AIDS". (KWON000008-9).
9. The work leading up to the identification and isolation of murine 4-1BB sequences was first disclosed in a U.S. patent application filed on November 7, 1988 (U.S. Ser. No. 07/267,577) (the "'577 application", EXHIBIT 4, KWON000010-41) and was then published in March 1989

Byoung S. Kwon

08/9-48,76-4

in PNAS (see Kwon et al., (1989), cDNA sequences of two inducible T-cell genes. *Proc Natl Acad Sci U S A.*; 86(6):1963-7. (Contributed on 10/24/88), EXHIBIT 5, KWON000042-46). In the '577 application (EXHIBIT 4), on page 18, line 38-page 19, line 13 (KWON000027-28), the specification describes one of the general methodologies behind isolating the human 4-1BB homolog using labeled mouse 4-1BB cDNA probe under various stringent conditions. Thus, isolation and characterization of the mouse 4-1BB gene provided the impetus for the subsequent isolation of its human counterpart.

10. Prior to May 7, 1993, I further characterized the murine 4-1BB gene to show that 4-1BB functions as a T-cell mediator. (e.g., Kwon et al., (1989) Expression characteristics of two potential T cell mediator genes., *Cell Immunol.*; 121(2):414-22, EXHIBIT 6, KWON000047-55). Demonstrating that 4-1BB functions as a T-cell mediator was an important step in elucidating the mechanistic basis for understanding the role of 4-1BB in immunology, thus providing a strong rationale for isolating the human homolog.

11. Prior to May 7, 1993, I raised antibodies against recombinant mouse 4-1BB protein products and also generated soluble forms of mouse 4-1BB for inhibition studies. (EXHIBIT 7, KWON000063).

12. My mouse 4-1BB research demonstrated that 4-1BB gene was present and played an important role in immune regulation. Therefore, based on my research with mouse 4-1BB gene and its gene product, I appreciated the overall importance of 4-1BB in T-cell activation and contemporaneously conceived of experiments to isolate the human homolog of 4-1BB. Thus, my research with the mouse 4-1BB gene and its gene product paved the way for subsequent isolation and characterization of the human homolog of 4-1BB.

13. Prior to May 7, 1993, hybridization of murine 4-1BB probe was performed on a northern blot with a variety of human tissue RNA that had been stimulated with Con A and PMA. (EXHIBIT 8, KWON000066). The northern blot shows a distinct band in the stimulated lanes indicating likely presence of a human homolog of 4-1BB mRNA. Subsequent efforts to clone the human homolog of 4-1BB gene through a probe hybridization-based approach were not helpful and therefore I adopted a degenerate primer-based approach that was primarily based on the mouse 4-1BB nucleotide sequence.

Byoung S. Kwon

08/948,764

14. Prior to May 7, 1993, relying on the mouse 4-1BB sequence and TNFR/NGFR superfamily members and their conserved domains, I designed degenerate primers to amplify specific regions of human 4-1BB nucleotide sequence. (EXHIBIT 9, KWON000067).

15. Prior to May 7, 1993, I designed degenerate primers that were capable of amplifying a portion of human 4-1BB. (EXHIBIT 10, KWON000069). The degenerate primers are represented as follows:

4-1BB-3F

5'- GAA TGT GAA TGT ATT GAA GG -3'  
           G   C    G    C    C    G  
                           A

16. The degenerate primer (4-1BB-3F) is the same as the degenerate primer H4-1BBF11 disclosed in the parent patent application U.S. Ser. No. 08/122,796 (the "'796 patent application") (EXHIBIT 12, KWON000087), filed September 16, 1993 and in the later published article Zhou et al., (1995), Characterization of human homologue of 4-1BB and its ligand, *Immunol Lett.*; 45(1-2):67-73 (EXHIBIT 13, KWON000106). The reverse degenerate primer is represented as follows:

4-1BB-3R

5'- GA5 AA5 GAA CA5 GTT TTA CA -3'  
           G   CTG           C    G

(KWON000068).

17. The degenerate primer (4-1BB-3R) is the same as degenerate primer H4-1BBR1 mentioned in the '796 patent application (EXHIBIT 12, KWON000087) and in Zhou et al., (1995), Characterization of human homologue of 4-1BB and its ligand, *Immunol Lett.*; 45(1-2):67-73 (EXHIBIT 13, KWON000106). The "5" in the primer sequence corresponds to "I" (inosine).

18. Prior to May 7, 1993 I designed the following degenerate primers (EXHIBIT 11, KWON000071):

4-1BB-1

5'- TTC TGT CG5 AAA TAT AAT CC -3'

Byoung S. Kwon

08/948,764

T C A G C C

19. The degenerate primer (4-1BB-1) is the same as degenerate primer H4-1BBF1 mentioned in the '796 patent application (EXHIBIT 12, KWON000087) and in Zhou et al., (1995), Characterization of human homologue of 4-1BB and its ligand, *Immunol Lett.*; 45(1-2):67-73 (EXHIBIT 13, KWON000106). The "5" in the primer sequence corresponds to "I" (inosine). The reverse degenerate primer is as follows:

4-1BB-2

5'- TTT TGA TCA TTA AA5 GT5 CC -3'  
C G G

(EXHIBIT 11, KWON000072).

20. The degenerate primer (4-1BB-2) is the same as degenerate primer H4-1BBRII mentioned in the '796 patent application (EXHIBIT 12, KWON000087) and in Zhou et al., (1995), Characterization of human homologue of 4-1BB and its ligand, *Immunol Lett.*; 45(1-2):67-73 (EXHIBIT 13, KWON000106). The "5" in the primer sequence corresponds to "I" (inosine).

21. Prior to May 7, 1993, the degenerate primers were successfully used to clone the human 4-1BB cDNA sequence as described in the '796 patent application. (EXHIBIT 12, KWON000086-88). mRNA isolated from human peripheral blood lymphocytes were activated with PMA (10 ng/ml) and ionomycin (1  $\mu$ M). The mRNA was converted to single-stranded cDNA using reverse transcriptase. The cDNA was then amplified with Taq polymerase with combination of the primers. The combination of primers was as follows: H4-1BBF1 vs H4-1BBRI; H4-1BBF1 vs H4-1BBRII; H4-1BBFII vs H4-1BBRI; and H4-1BBFII vs H4-1BBRII. (EXHIBIT 12, KWON000087).

22. Thus, prior to May 7, 1993, as evidenced by the '796 patent application (EXHIBIT 12) and in the Zhou et al. (1995) publication (EXHIBIT 13), the primer pair — H4-1BBFII (4-1BB-3F) and H4-1BBRII (4-1BB-2) produced bands of about 240 bp. The 240 bp was the expected size of human 4-1BB based on the mouse 4-1BB sequence. The PCR product (240 bp) libraries were screened in a low stringent condition with murine 4-1BB cDNA and positive clones were

Byoung S. Kwon

08/948,764

subcloned in a PGEM3 vector and sequenced. An open reading frame of the PCR product was about 65% identical to mouse 4-1BB and it was therefore established that the 240 bp PCR product contained the human homolog of mouse 4-1BB. The 240 bp PCR product was then used to screen a  $\lambda$ gt11 cDNA library of activated human T lymphocytes, from which a 0.85 kb cDNA fragment was isolated. The sequence of the human 4-1BB cDNA is shown in FIG. 2a and the predicted amino acid sequence is shown in FIG. 2b of the '796 patent application. (EXHIBIT 12, KWON000101).

23. I was interested in studying the role of human 4-1BB, especially it's role in T-cell co-stimulation, as evidenced by the published work regarding murine 4-1BB e.g., in Pollok *et al.*, (1993), Inducible T Cell Antigen 4-1 BB, *J. Immunology*, 150: 771-781 (received for publication October 30, 1991). (EXHIBIT 14, KWON000112-122). In an effort to understand the functions of human 4-1BB, similar to my earlier work with the mouse 4-1BB, I designed experiments that involved the use of recombinant human 4-1BB including fusion proteins of human 4-1BB. As part of this research, I designed primers to clone recombinant fusion nucleotide sequences of human 4-1BB based on the full-length sequence of human 4-1BB cDNA.

24. Prior to May 7, 1993, based on the full-length sequence of the human 4-1BB I designed specific primers to generate recombinant fusion proteins of human 4-1BB. The following primers were designed. (EXHIBIT 15, KWON000123):

Forward:	AAT AAG CTT TGC TAG TAT CAT ACC T
Reverse for plg:	TTA AGA TCT CTG CGG AGA GTG TCC TGG CTC
Reverse for full:	GGA ATT CCA GCT CTG TCC TCA TCT GTC T

25. Prior to May 7, 1993, as demonstrated in EXHIBIT 15 and as shown in the illustration to EXHIBIT 15 (KWON000124) and the description in the '796 application (EXHIBIT 12, KWON000088-89), the 5'-portion of the human 4-1BB cDNA that included the sequence encoding the signal sequence and the extracellular domain, was amplified by PCR. For directional cloning purposes, a HindIII site on the 5' end of the forward primer and a BglII site on the 5' end of the reverse primer were created. (EXHIBIT 15, KWON000124).

Byoung S. Kwon

08/948,764

26. The forward primer (5'-AAT AAG CTT TGC TAG TAT CAT ACC T-3') starts upstream of the human 4-1BB coding sequence. The reverse primer (5'-TTA AGA TCT CTG CGG AGA GTG TCC TGG CTC-3') was used for cloning a portion of H4-1BB into a Ptag vector. The reverse primer for the full length human 4-1BB (5'-GGA ATT CCA GCT CTG TCC TCA TCT GTC T-3') starts downstream of the stop codon. The relative locations of the primer sequences with respect to the full-length human 4-1BB sequence are provided. (EXHIBIT 15, KWON000124). The human 4-1BB matching regions in the primers are underlined and the corresponding portions in human 4-1BB are highlighted. (EXHIBIT 15, KWON000124). The restriction sites are underlined. AAG CTT corresponds to Hind III; AGA TCT corresponds to BglII; and GAA TTC corresponds to EcoR I restriction sites.

27. Thus, Prior to May 7, 1993, clones containing the full-length human 4-1BB were sequenced and the full-length cDNA sequence was available in my laboratory for further experiments. I designed specific primers based on the full-length sequence to amplify human 4-1BB fragments for directional cloning into expression vectors. For example, Ptag was a common expression vector with an epitope tag placed adjacent to a multiple cloning site for effective identification of the expressed recombinant protein.

28. Prior to May 7, 1993, under my direction and guidance, Dr. Kack Kim, a visiting scientist at my laboratory, performed some of the cloning experiments involving human 4-1BB and a human placental alkaline phosphatase tag (APtag-1) expression vector. (EXHIBIT 16, KWON000125-128). The alkaline phosphatase-4-1BB fusion protein was useful in determining the relative amount of the 4-1BB protein present in the various cell types. (EXHIBIT 13, KWON000110). The APtag vector and the human 4-1BB PCR products were digested with restriction enzymes BglII and HindIII for directional cloning as described earlier in the design of primers. (EXHIBIT 15, KWON000123-124). As shown in the gel picture of EXHIBIT 16 (KWON000128), APtag vector and human 4-1BB fragment digested with BglII and HindIII show distinct bands for the lanes marked as APtag/HindIII-BglII and 4-1BB (PCR)/ Hind III-BglII. The restricted vector and the PCR product (HindIII-BglII 4-1BB fragment) were gel purified and ligated with a ligase. (EXHIBIT 16, KWON000125-127). The human 4-1BB fragment that included the extracellular domain was cloned upstream of the coding sequence of the alkaline phosphatase in the mammalian expression vector. (EXHIBIT 13, KWON000106).

*Byoung S. Kwon*

08/948,764

The resulting plasmid was designated 4-1BB-AP as mentioned in EXHIBIT 13 (KWON000106) and EXHIBIT 12 (KWON000088). To obtain the fusion protein, the 4-1BB-AP plasmid was linearized with the restriction enzyme *Cl*I and was co-transfected with a linearized selectable marker plasmid, pSV7neo, by calcium phosphate co-precipitation method. (EXHIBIT 13, KWON000106). Alkaline phosphatase assay was used to select the clones that produced the highest levels of human 4-1BB-AP fusion protein. (EXHIBIT 13, KWON000106).

29. Prior to May 7, 1993, the A<sup>+</sup>tag-human 4-1BB clone (4-1BB-AP) was sequenced to verify the in-frame fusion of the extracellular domain of human 4-1BB with the alkaline phosphatase of the A<sup>+</sup>tag vector and was stored in my laboratory freezer as evidenced by a log book entry made by Todd Pickard, a technician in my laboratory at that time. (EXHIBIT 17, KWON000131). The freezer log-book was maintained as part of routine lab-keeping to generate a catalog of cultures, plasmids, vectors, probes and other stocks that were stored in my laboratory freezer. The log book entry demonstrates that the vector expressing the human 4-1BB-AP fusion protein was generated and its sequence was confirmed by sequencing prior to May 7, 1993. Therefore, prior to May 7, 1993, a vector that encoded a recombinant fusion protein of a fragment of human 4-1BB was generated. Purification of proteins expressed from an expression vector was readily and routinely practiced in my lab prior to May 7, 1993.

30. Prior to May 7, 1993, an *Eco*RI digested human 4-1BB fragment was ligated with a mammalian expression vector pXM that was also digested with *Eco*RI and treated with calf-intestinal phosphatase (CIP) to reduce vector self-ligation. (EXHIBIT 18, KWON000158-160, and KWON000166). pXM was a mammalian expression vector that was routinely used for insert cloning and expression for example, in mammalian cells such as COS-1 cell line, when an expression construct with a suitable promoter is introduced into the COS cells.

31. In addition to generating fusion proteins of human 4-1BB, prior to May 7, 1993, homology analysis of 4-1BB in other species was performed. (EXHIBIT 18, KWON000130). Prior to May 7, 1993, a human 4-1BB fragment was used as a probe to hybridize blots containing DNA isolated from a variety of species including mouse, gibbon, and human DNA that were restricted with *Eco*RI (EXHIBIT 18, KWON000130-132). In addition, amplifications of total and polyA RNA using human 4-1BB primers were also performed. MLA polyA<sup>+</sup> refers to



Byoung S. Kwon

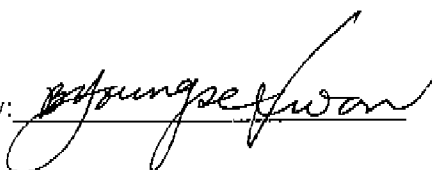
08/948,764

Gibbon T-cell RNA and Jurkat and Molt4 refer to T-cell lines of human origin. Prior to May 7, 1993, a 1.2 Kb fragment of human 4-1BB DNA was labeled with radioactive ATP and CTP by a nick-translation method. (EXHIBIT 18, KWON000142-143). The labeled human 4-1BB probe was then used to hybridize Southern blots containing DNA from mouse, Gibbon and human. The hybridization was at high stringency and carried over at 65°C over night. After a high-stringency washing, the blot was exposed for 4 days. Prior to May 7, 1993 dot blot of PCR products from the amplification of MLA total/polyA+ RNA was probed with labeled human 4-1BB fragment. (EXHIBIT 18, KWON000145-149).

32. In summary, prior to May 7, 1993, I designed degenerate primers to obtain human 4-1BB cDNA sequence, used the full-length human 4-1BB cDNA sequence information to design specific primers for directional cloning of a human 4-1BB DNA fragment in an expression vector, and under my guidance and supervision, the DNA encoding human 4-1BB protein including the extracellular domain was cloned and the protein product was purified for subsequent analysis.

33. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that those statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: Sept. 2, 2008

By:   
Byoung S. Kwon